Enterococcus thailandicus sp. nov., isolated from fermented sausage ('mum') in Thailand

Somboon Tanasupawat,¹ Sirapan Sukontasing¹ and Jung-Sook Lee²

Correspondence

Somboon Tanasupawat

Somboon.T@chula.ac.th

¹Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

²Korean Collection for Type Cultures (KCTC), Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 52 Eoeun-dong, Yuseong-gu, Daejeon 305-333, Republic of Korea

A Gram-positive and catalase-negative coccus that formed chains, designated strain FP48-3^T, isolated from fermented sausage ('mum'), was studied systematically. Strain FP48-3^T was facultatively anaerobic and produced L-lactic acid from glucose. Straight-chain fatty acids C_{18:1} and C_{16:0} were the dominant components. The DNA G + C content of strain FP48-3^T was 37.9 mol%. On the basis of 16S rRNA and RNA polymerase α -subunit (*rpoA*) gene sequence analysis, strain FP48-3^T was closely related to *Enterococcus hirae* LMG 6399^T, *Enterococcus durans* LMG 10746^T and *Enterococcus faecium* LMG 11423^T, with 99.3–99.6 and 95.1–96.9 % sequence similarities, respectively. Strain FP48-3^T could be clearly distinguished from *E. hirae* LMG 6399^T, *E. durans* LMG 10746^T and *E. faecium* LMG 11423^T by low DNA–DNA relatedness (≤ 14 %) and phenotypic characteristics. Therefore, this strain represents a novel species of the genus *Enterococcus*, for which the name *Enterococcus thailandicus* sp. nov. is proposed. The type strain is FP48-3^T (=KCTC 13134^T=NBRC 101867^T=NRIC 0107^T=TISTR 933^T=PCU 282^T).

Enterococci have been isolated from plants, soil, animals, humans and raw and prepared food including meat, poultry, fish, egg, milk, cheese and vegetables and traditional fermented foods (Knudtson & Hartman, 1992; Sukontasing *et al.*, 2007). The classification of the genus *Enterococcus* has undergone considerable changes as a consequence of the increase in the number of novel species and also improvements in the methods used to discriminate separate species (Baele *et al.*, 2000; Merquior *et al.*, 1994; Naser *et al.*, 2005). At the time of writing, 34 *Enterococcus* species names have been validly published (Euzéby, 1997; last full update May 14, 2008). Several species groups, *Enterococcus faecium*, *E. avium*, *E. gallinarum*, *E. cecorum*, *E. faecalis* and other species groups, including *E. saccharolyticus*, *E. sulfureus* and *E. dispar*, were revealed based on comparative 16S rRNA gene sequence analysis (Hardie & Whiley, 1997; Švec *et al.*, 2006). Here we describe a novel bacterium, strain FP48-3^T, isolated from fermented sausage ('mum'), and propose that it represents

a novel species, based on phenotypic and chemotaxonomic characteristics, DNA–DNA relatedness and 16S rRNA and RNA polymerase α -subunit (*rpoA*) gene sequence analysis.

Samples of fermented sausage were collected from Khonkaen province, north-eastern Thailand. Cocci arranged in chains were isolated from the samples using GYP-CaCO₃ agar (Tanasupawat *et al.*, 1992). Cultures grown using GYP-sodium acetate-mineral salt broth (Tanasupawat *et al.*, 1992), adjusted to pH 7.2, were used for working cultures. All tests were performed by incubating the cultures at 30 °C. Cell shape, size, arrangement and colony appearance were examined using cells grown on GYP agar for 3 days. Gram staining was done as described by Hucker & Conn (1923). Spore formation was examined using the Gram-stained specimen. Results of the oxidation–fermentation test and motility were examined in soft agar (Whittenbury, 1963). Catalase activity, hydrolysis of gelatin, aesculin, arginine and starch, nitrate reduction, production of gas from glucose, gluconate and citrate, and acid formation from carbohydrates were tested as reported by Tanasupawat *et al.* (1992). Additional biochemical characteristics were recorded after 2 days incubation in API 50 CH strips. Hydrolysis of horse blood was assessed as described by Barrow & Feltham (1993). Growth on Slanetz–Bartley agar (Oxoid) and kanamycin aesculin azide agar (Merck) was tested. The

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoA* gene sequences of strain FP48-3^T are EF197994 and EU015878, respectively.

Neighbour-joining phylogenetic trees, based on 16S rRNA and *rpoA* gene sequences, showing the relationships between strain FP48-3^T and related species are available as supplementary material with the online version of this paper.

reaction in litmus milk (Difco) was investigated after incubating cultures for 3, 7 and 14 days. The effects of temperature (10–45 °C), starting pH (4.0, 4.5, 5.0, 7.5, 9.0 and 9.6) and NaCl concentration (2, 4, 6, 6.5, 8 and 10 %, w/v) were determined by examining growth in GYP-sodium acetate-mineral salt broth. Vitamin requirements were examined using the method of Kihara & Snell (1960) with modifications. The isomer of lactic acid was analysed enzymically (Okada *et al.*, 1978). Fatty acid methyl esters were prepared as described by Ikemoto *et al.* (1978) and cellular fatty acid compositions were analysed by using GLC [model GC-14A (Shimadzu), equipped with a CBP1 (OV-1) type capillary column, 25 m × 0.25 mm inside diameter, at 180–220 °C and a flame ionization detector]. Gas-liquid chromatograms were calculated by using the Chromatopac C-R 4A data-processor (Shimadzu). Quinones were extracted from freeze-dried cells and purified as described by Collins *et al.* (1977) and Collins

& Jones (1979). The purified quinones were analysed by HPLC (Tamaoka *et al.*, 1983).

DNA was isolated and purified using the method of Saito & Miura (1963). The DNA base composition was determined using reversed-phase HPLC (Tamaoka & Komagata, 1984). PCR amplification and sequencing of the 16S rRNA and *rpoA* genes were performed as described by Lane (1991) and Naser *et al.* (2005), respectively. The sequences were assembled and compared with deposited type strain sequences available in GenBank/EMBL/DDBJ by using BioEdit version 7.0.1 (Hall, 1999) and CLUSTAL_X version 1.83 (Thompson *et al.*, 1997). Phylogenetic trees were constructed based on the maximum-likelihood (Felsenstein, 1989) and the neighbour-joining (Saitou & Nei, 1987) methods by using the program NJPlot (Perrière & Gouy, 1996). Confidence values of branches of the phylogenetic trees were determined using bootstrap

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain FP48-3^T and related bacterial species. The branching pattern was generated by using the maximum-likelihood method. Based on 1000 replications, bootstrap percentages ≥ 51 % are shown. Bar, 0.01 substitutions per nucleotide position.

analysis (Felsenstein, 1985) based on 1000 resamplings. DNA–DNA hybridization experiments were performed as reported by Ezaki *et al.* (1989).

Cells of strain FP48-3^T were Gram-positive, facultatively anaerobic, non-motile, non-spore-forming, spherical or ovoid, and arranged in pairs or in chains. Detailed morphological, cultural, physiological and biochemical properties, including chemotaxonomic characteristics, are given in the species description. Menaquinones were not detected. Strain FP48-3^T contained the straight-chain fatty acids C_{18:1} (45.9 %) and C_{16:0} (25.7 %) as the dominant components. The remainder of the fatty acids profile consisted of C_{14:1} (1.2 %), C_{14:0} (10.1 %), C_{16:1} (13.3 %), a trace amount of C_{18:0} and unidentified components (2.4 %). Strain FP48-3^T contained roughly the same fatty acid pattern as its closest relative, *E. hirae* LMG 6399^T. The fatty acid C_{20:1} and cyclopropane acids C₁₇ and C₁₉ were not detected in strain FP48-3^T, but were present in *E. hirae* LMG 6399^T. The DNA G + C content of strain FP48-3^T was 37.9 mol%. The almost-complete 16S rRNA gene sequence (1326 bp) of strain FP48-3^T indicated that the strain

belonged to the genus *Enterococcus* and was closely related to *E. hirae* LMG 6399^T (99.6 %), *E. durans* LMG 10746^T (99.6 %) and *E. faecium* LMG 11423^T (99.3 %). Lower sequence similarities (<98.7 %) were found with other recognized species of the genus *Enterococcus* (Fig. 1). A tree based on 16S rRNA gene sequences, constructed using the neighbour-joining method, is available as Supplementary Fig. S1, in IJSEM Online. Strain FP48-3^T was included in the *E. faecium* species group that currently contains *E. faecium*, *E. hirae*, *E. durans*, *E. mundtii*, *E. villorum*, *E. ratti* and *E. canis* (Collins *et al.*, 1984, 1986; Farrow & Collins, 1985; Vancanneyt *et al.*, 2001; Teixeira *et al.*, 2001; De Graef *et al.*, 2003). The *rpoA* gene sequence (615 bp) of strain FP48-3^T showed 96.9, 95.6 and 95.1 % similarity to *E. faecium* LMG 11423^T, *E. durans* LMG 10746^T and *E. hirae* LMG 6399^T, respectively (Fig. 2). A tree constructed using the neighbour-joining method is available as Supplementary Fig. S2, in IJSEM Online. The application of multilocus sequence analysis for rapid identification of *Enterococcus* species based on the *rpoA* gene (Naser *et al.*, 2005, 2006), confirmed the separation of strain FP48-3^T from related species. Strain FP48-3^T showed low

Fig. 2. Phylogenetic tree based on *rpoA* gene sequences, showing the relationships between strain FP48-3^T and related bacterial species. The branching pattern was generated by using the maximum maximum-likelihood method. Based on 1000 replications, bootstrap percentages ≥ 57 % are shown. Bar, 0.01 substitutions per nucleotide position.

DNA–DNA relatedness to *E. hirae* LMG 6399^T (6%), *E. durans* LMG 10746^T (7%), *E. faecium* LMG 11423^T (14%) and *E. mundtii* LMG 10748^T (6%). In addition, strain FP48-3^T could be differentiated from closely related *Enterococcus* species by growth at pH 5 and no growth at 10 °C, acid production and DNA G+C content, as shown in Table 1. Therefore, strain FP48-3^T should be classified in the genus *Enterococcus* as representing a novel species, *Enterococcus thailandicus* sp. nov.

Description of *Enterococcus thailandicus* sp. nov.

Enterococcus thailandicus (tha.i.lan'di.cus. N.L. masc. adj. *thailandicus* of Thailand, pertaining to Thailand, where the type strain was isolated).

Cells are Gram-positive, facultatively anaerobic, non-motile, non-spore-forming, spherical or ovoid, 0.5–1 µm in diameter and arranged in pairs or in chains. Colonies on GYP agar are circular, raised or low-convex with entire margins, and non-pigmented. Red colonies appear on Slanetz–Bartley agar. Grows on kanamycin aesculin azide agar. Positive for hydrolysis of arginine and aesculin, and weakly positive for blood haemolysis. Negative for catalase, hydrolysis of gelatin and starch, reduction of nitrate, and production of gas from glucose, gluconate and citrate. Utilizes glucose fermentatively. No acidification, coagulation, reduction or liquefaction in litmus milk. Grows at pH 5.0–9.6, 15–45 °C and in 2–6.5% NaCl. Weak growth occurs in 8% NaCl. Acid is produced from D-amydalin, arbutin, cellobiose, dulcitol, fructose, D-galactose, β-gentiobiose, gluconate, D-glucose, glycerol, N-acetylglucosamine, lactose, maltose, D-mannose, methyl α-D-mannoside, D-mannitol, D-ribose, sucrose and salicin but not from adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, erythritol, D-fucose, L-fucose, 2-ketogluconate,

5-ketogluconate, methyl α-D-glucoside, glycogen, inositol, inulin, D-lyxose, melezitose, melibiose, rhamnose, L-sorbose, starch, turanose, D-tagatose, D-sorbitol, raffinose, trehalose, xylitol, D-xylose, L-xylose or methyl β-xyloside. Niacin, calcium pantothenate and folic acid are required for growth. Does not contain any menaquinones. The dominant component of the fatty acid profile is the straight-chain acid C_{18:1}. The DNA G+C content of the type strain is 37.9 mol%.

The type strain, FP48-3^T (=KCTC 13134^T=NBRC 101867^T=NRIC 0107^T=TISTR 933^T=PCU 282^T), was isolated from fermented sausage ('mum'), produced in Thailand.

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Table 1. Differential characteristics of strain FP48-3^T (*E. thailandicus* sp. nov.) and related *Enterococcus* species

Strains: 1, FP48-3^T; 2, *E. hirae* LMG 6399^T; 3, *E. durans* LMG 10746^T; 4, *E. faecium* LMG 11423^T. +, Positive; –, negative.

Characteristic	1	2	3	4
Growth at:				
pH 5.0	+	–	+*	+
10 °C	–	+	+*	+
Acid production from:				
D-Galactose	+	–	+	+
Gluconate	+	–	–	+
Glycerol	+	–	–	+
D-Mannitol	+	+	–	+
Melibiose	–	+	–	–
Salicin	+	–	+	+
D-Sorbitol	–	+	–	–
DNA G+C content (mol%)	37.9	38.0	38.6	39.0

*Data from Cai et al. (1999).

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